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Ninth day of April 2004

J. Billingsley

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WOMEN'S AND CHILDREN'S HOSPITAL

AUSTRALIA

PATENTS ACT 1990

PROVISIONAL SPECIFICATION FOR AN INVENTION ENTITLED:-

"AN IMPROVED METHOD OF SCREENING FOR LYSOSOMAL STORAGE DISORDERS (LSD)"

This invention is described in the following statement:-

FIELD OF THE INVENTION

This invention relates to an improved method for screening individuals for Lysosomal Storage Disorders (LSD).

BACKGROUND

LSD represent a group of over 40 distinct genetic diseases that generally become apparent when an affected child is young and have a devastating impact on the child and the family. Affected individuals can present with a wide range of clinical symptoms depending upon the specific disorder and the particular genotype involved. Central nervous system dysfunction, from behavioural problems to severe mental retardation, is characteristic of many LSD.

In severe cases, the child requires constant medical management but often dies before adolescence. The significance of LSD to health care becomes obvious when the group incidence rate for LSD (1:5,000 births) is compared with well-known and intensively studied genetic disorders such as phenylketonuria (1:14,000) and cystic fibrosis (1:2,500).

Over the past 20 years there has been considerable progress in the diagnosis of LSD presenting clinically. However, except for those cases with a family history of the disease, pre-symptomatic detection of LSD can only be achieved by newborn screening. Currently, diagnosis of LSD is a complex process involving a range of assays performed on purified leucocytes, cultured skin fibroblasts or biopsy material. Individuals having LSD are screened/diagnosed on the basis of a decrease in the activity of the specific enzyme deficient in that disorder. These assays are typically performed on the enzyme activity of more than 40 enzymes involved to identify which specific LSD is present in the individual. As the skilled person in the art would appreciate, this leads to a lengthy and expensive time frame before the individual is accurately diagnosed with a specific LSD.

The prior art teaches the use of assays that measure enzyme activity (see Table 1 on pages 7-8 for a complete list at the present time), and there have been reports that

the majority of LSDs result from a reduction of the amount of the enzyme protein involved in the specific LSD and that these amounts may assist in screening assays (Isbrandt et al 1994; Umapathysivam et al 2001).

One common feature of LSD is the accumulation and storage of material within lysosomes. This material is normally degraded within the lysosome; and the products of degradation are usually transported across the lysosomal membrane. It is generally recognised that the accumulation and storage of material results in an increase in the number and size of lysosomes within the cell from approximately 1% to as much as 50% of total cellular volume. The inventors have previously found that the level of certain lysosomal proteins would be elevated as a result of storage and that these proteins may prove to be useful biomarkers for the detection of all LSD [Meikle et al 1997; Hua et al 1998]. The lysosome-associated membrane proteins (LAMPs), the saposins and α-glucosidase have been identified as useful biomarkers.

SUMMARY OF THE INVENTION

The inventors have identified that current screening assays are largely inaccurate because they do not take into consideration the variations in white blood cell counts between individuals.

The inventors have subsequently shown that the accuracy of the screening process for LSD is improved when the enzyme activity or protein levels are normalised against all lysosomal proteins, enzymes or enzyme activities, or a combination thereof. It will be appreciated by those skilled in the art that this principle could be used to improve the accuracy of screening processes for other diseases.

The following are examples of the main lysosomal proteins that would be used as normalising reference markers:

- ⇒ LAMP-1 or LAMP-2; or
- ⇒ Saposin A, B, C or D; or
- ⇒ α-Glucosidase, α-L-Iduronidase, Iduronate-2-sulphatase, N-

Acetylgalactosamine 4-sulphatase, Galactose 6-sulphatase, Acid sphingomyelinase, Galactocerebrosidase, Arylsulphatase A, Heparan-N-sulphatase, α-N-Acetylglucosaminidase, β-Galactosidase, β-Hexosaminidase A or β-Hexosaminidase B; or

⇒ a combination of any of the above markers.

It is proposed that any assay which enables the simultaneous determination of multiple proteins or enzyme activities from a single patient sample. (eg Multiplex Assay) can be used.

Furthermore, it is proposed that grouping these lysosomal proteins, enzymes or enzyme activities may give a fingerprint pattern of the health of lysosomal organelles in an individual (eg newborns). This may assist in identification/prediction of the type of LSD. For example, Mucolipidosis type II and multiple sulphatase deficiency would be expected to have characteristic fingerprints that are different from that of a single enzyme deficiency such as Gaucher. Further, Gaucher may generate a fingerprint character that will support/confirm the suspicion that an individual with a reduced beta-glucosidase activity [the enzyme involved in Gaucher] has this LSD.

Accordingly, there is provided a method of screening individuals for LSD wherein the enzyme activity or protein level characteristic of the LSD is normalised against the level of a lysosomal protein, enzyme or enzyme activity which is unaffected by the LSD, and hence is adjusted to take into account differing numbers of lysosomes between individuals.

In another aspect of the invention, there is provided a method of determining the "fingerprint" pattern for the health of lysosomal organelles in an individual. A "fingerprint" is an individual pattern obtained from grouping lysosomal protein or activity data from a number (greater than two) of different lysosomal enzymes/proteins. The "fingerprint" pattern of an individual can enable the identification of a specific LSD and the clinical severity of the LSD.

It is preferred that, in both methods, a single assay (eg Multiplex) is used to measure protein or enzyme levels, enzymatic activities, or a combination thereof.

DESCRIPTION OF THE INVENTION

Experimental methods: The BioPlex Protein Array System (BioRad) was used to establish proof of the invention, using three assay systems. The assay system used in the following is called the Multiplex Assay System and allows the simultaneous determination of multiple analytes from a single sample. The multiplex process is detailed below:

1. Preparation of multiplex reagents

The capture antibodies (monoclonal and sheep polyclonal) were coupled to polystyrene microspheres using the BioRad bead coupling kit. Protein (antibody) coupling was validated according to the manufacturer's protocol.

Coupling of the phycoerythrin reporter molecule to the detection antibodies in the LAMP-1, saposin C and α-glucosidase assays was achieved using the Molecular Probes (Eugene Oregon USA) Protein-Protein Coupling Kit, as per manufacturer's instructions.

2. Development of multiplex assays

The assays for LAMP-1, saposin C and α-glucosidase were multiplexed using the bead sets 17, 19, and 21 (BioRad, Hercules, CA, USA) to demonstrate the value of the concept of multiplexing.

Standard curves using liquid calibrators were performed in both a single assay format (Figure 1) and a multiplex of 3 (Figure 2).

3. Proposed multiplex method to screen the newborn population for major LSD

The following is a theoretical example of a single Multiplex assay that could be used in North America and Europe. Twelve LSD (in brackets, the following list) were chosen because of their relatively high prevalence in North America and Europe, together with the availability of effective therapies that would benefit from early diagnosis. The Multiplex could, for example, test for the following 14 proteins (associated LSD in brackets): LAMP-1 (generic LSD), saposin C (generic LSD), α-glucosidase (Pompe), α-galactosidase A (Fabry), glucocerebrosidase or β-glucosidase (Gaucher), α-iduronidase (MPS I), iduronate-2-sulphatase (MPS II), Heparan-N-sulphatase (MPS IIIA), α-N-acetylglucosaminidase (MPS IIIB), galactose-6-sulphatase (MPS IVA), β-galactosidase or galactocerebrosidase (Krabbe), galactose-3-sulphatase (MLD), sphingomyelinase (Niemann-Pick A/B) and N-acetylgalactosamine-4-sulphatase (MPS VI).

The multiplex technology will enable the combination of LSD screened for to be changed as treatment methods improve, new LSD are identified or needs in different geographic areas change.

The person skilled in the art would appreciate that antibodies to all of the proposed 14 proteins would be required.

The present invention improves the accuracy and detection of all of these LSD and can for example be completed in a single multiplex assay. LAMP-1 and saposin C are used as markers to normalise the population for the lysosomal content of the patient sample. For some disorders, these proteins may provide additional discriminatory power by showing an increase in concentration relative to the non disease state. By calculating the ratio of these proteins to the individual proteins deficient in each LSD, greater discriminatory power can be attained. This concept can be extended beyond the calculation of ratios of individual proteins to the determination of protein profiles which look at all protein concentrations determined for a given sample. The use of discriminate analysis or other statistical

methods can provide improved discrimination between control and affected populations.

It is to be understood that proteins characteristic of other LSD types can replace, or add to, the 14 lysosomal proteins listed above and that such modifications may depend on the frequency of individual LSD for particular geographic regions. For example, the relative prevalence of individual LSD is different in North America, Japan and China.

Table 1 Enzymes deficient in some common lysosomal storage disorders

Disease	Clinical Phenotype	Enzyme Deficiency	Australian Prevalence
Gaucher disease types I / II / III	Gaucher disease	Glucocerebrosidase (β-glucosidase)	1 in 57,000
Cystinosis		Cystine transporter	1 in 192,000
Fabry disease	Fabry disease	α-Galactosidase A	1 in 117,000
Glycogen storage disease II	Pompe disease	α-Glucosidase	1 in 146,000
Mucopolysaccharidosis type I	Hurler/Scheie syndrome	α-L-Iduronidase	1 in 88,000
Mucopolysaccharidosis type II	Hunter syndrome	Iduronate-2-sulphatase	1 in 136,000
Mucopolysaccharidosis type VI	Maroteaux-Lamy syndrome	N-Acetylglucosamine 4-sulphatase	1 in 235,000
Mucopolysaccharidosis type IVA	Morquio syndrome	Galactose 6-sulphatase	1 in 169,000
Niemann-Pick disease types A / B	Niemann-Pick disease	Acid sphingomyelinase	1 in 248,000
Globoid cell leucodystrophy	Krabbe disease	Galactocerebrosidase	1 in 201,000
Metachromatic leucodystrophy		Arylsulphatase A	1 in 92,000
Metachromatic leucodystrophy		Saposin B	
Mucopolysaccharidosis type IIIA	Sanfilippo syndrome	Heparan-N-sulphatase	1 in 114,000
Mucopolysaccharidosis type IIIB	Sanfilippo syndrome	α-N-Acetylglucosaminidase	1 in 211,000
Mucopolysaccharidosis type IIIC	Sanfilippo syndrome	AcetylCoA:N-acetyltransferase	1 in 1,407,000
Mucopolysaccharidosis type IIID	Sanfilippo syndrome	N-Acetylglucosamine 6-sulphatase	1 in 1,056,000
Mucopolysaccharidosis type IVB	Morquio syndrome	β-Galactosidase	
Mucopolysaccharidosis type VII	Sly	β-Glucuronidase	1 in 2,111,000
Niemann-Pick disease type C1	Niemann-Pick disease	Cholesterol trafficking	1 in 211,000
Niemann-Pick disease type C2	Niemann-Pick disease	Cholesterol trafficking	
Aspartylglucosaminuria		Aspartylglucosaminidase	1 in 2,111,000
Cholesterol ester storage disease	Wolman disease	Acid lipase	1 in 528,000
GM1-Gangliosidosis types I/II/III		β-Galactosidase	1 in 384,000
GM2-Gangliosidosis type I	Tay Sachs disease	β-Hexosaminidase A	1 in 201,000
GM2-Gangliosidosis type II	Sandhoff disease	β-Hexosaminidase A & B	1 in 384,000
GM2-Gangliosidosis		GM2-activator deficiency	
Farber Lipogranulomatosis	Farber disease	Acid ceramidase	
Fucosidosis		α-L-Fucosidase	> 1 in 2,000,000
Galactosialidosis types I / II		Protective protein	
α-Mannosidosis types I / II		α-D-Mannosidase	1 in 1,056,000
β-Mannosidosis		β-D-Mannosidase	
Mucolipidosis type I	Sialidosis types I / II	Neuraminidase	
Mucolipidosis types II / III	I-cell disease;	Phosphotransferase	1 in 325,000
Mucolipidosis type IIIC	pseudo-Hurler polydystrophy	Phosphotransferase g-subunit	

Mucolipidosis type IV		Unknown	
Multiple sulphatase deficiency		Multiple sulphatases	1 in 1,407,000
Neuronal Ceroid Lipofuscinosis, CLN1	Batten disease	Palmitoyl protein thioesterase	
Neuronal Ceroid Lipofuscinosis, CLN2	Batten disease	Tripeptidyl peptidase I	
Neuronal Ceroid Lipofuscinosis, CLN3	Vogt-Spielmeyer disease	Protein function not known	
Neuronal Ceroid Lipofuscinosis, CLN5	Batten disease	Protein function not known	
Neuronal Ceroid Lipofuscinosis, CLN8	Northern Epilepsy	Protein function not known	
Pycnodysostosis		Cathepsin K	
Sialic acid storage disease	Schindler disease	α-Galactosidase B	
Sialic acid storage disease	Sialuria; salla disease	Sialic acid transporter	1 in 528,000

Prevalence figures quoted from Miekle et al., *JAMA* 281:249-254 (1999). Prevalence and ratio of lysosomal storage disorders may vary from country to country

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DATED this 31st day of March, 2003.

WOMEN'S AND CHILDREN'S HOSPITAL
By its Patent Attorneys
MADDERNS

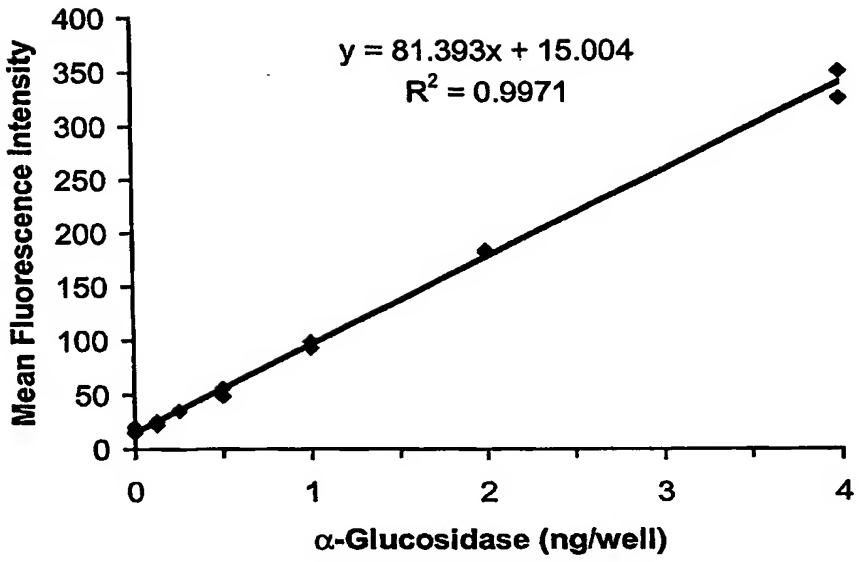

Figure 1 Calibration Curve for α-Glucosidase in a Microsphere Based Assay.
A calibration curve was generated using liquid calibrators in a microsphere based assay. Single assay not multiplexed. Mean Fluorescent intensity is the average of the total fluorescence detected for all beads in the defined bead region.

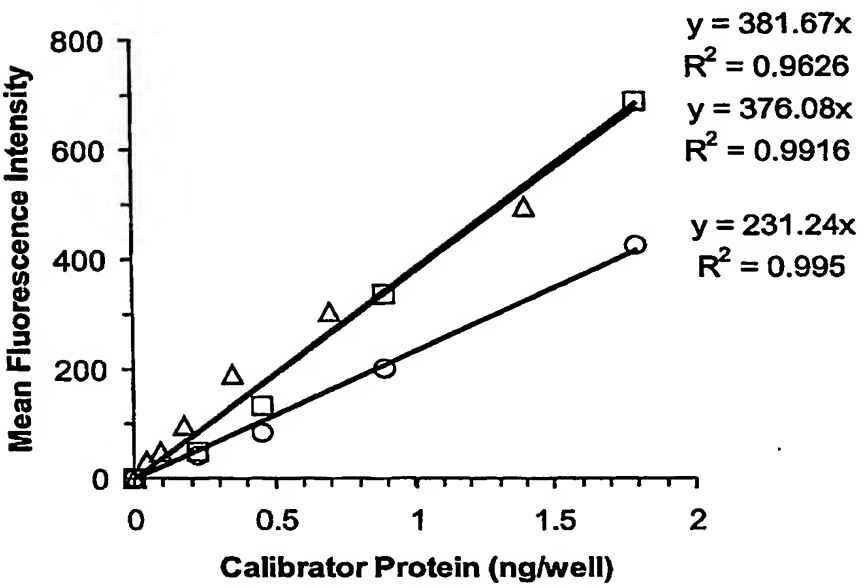

Figure 2. Multiplexed Calibration Curves in a Microsphere Based Assay. Calibration curves were established, using liquid calibrators, for Lamp-1 (open square), saposin C (open circle) and α-glucosidase (open triangle). Increased mean fluorescence intensity for the α-glucosidase protein (compared to Figure 1) is the result of improvements in the antibody labelling of the microspheres and the phycoerythrin labelled antibodies.